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Respiration induces variable porosity to polyols in the mitochondrial inner membrane

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The osmotic basis of low and high amplitude swelling in mitochondria was investigated in detail using sucrose and mannitol as external osmolytes. Osmotic behaviour of mitochondria in various respiratory states was consistent with significant changes in the porosity of the inner membrane corresponding to the rate of respiration. The stoichiometry of oxidative phosphorylation was confirmed to be dependent on the physical state (i.e., osmotic stretch) of the inner membrane regardless of the external polyol used. High amplitude swelling in polyol media was shown to arise from a sequential disruption of the outer and inner mitochondrial membranes, due to a dynamic instability induced by a combination of respiration, unscreened (fixed) surface charge density and the consequent variable porosity of the inner membrane. These novel experimental findings based on the physical theory of osmosis emphasize the need to define the fine structural changes of the inner membrane associated with oxidative phosphorylation to arrive at a comprehensive mechanism.

Introduction

The marked nonadiabatic behaviour of mitochondria, as manifest by relatively rapid contraction-swelling cycles in isotonic media associated with oxidative phosphorylation (low amplitude swelling) or due to respiration in nonelectrolyte media (high amplitude swelling) [1,2], remains a major unsolved paradox. The paradox lies in that, swelling (and/or contraction) and an invariant isotonicity (i.e., invariant permeability characteristics of the inner membrane) cannot coexist in a membrane-bound organelle that otherwise behaves as a perfect osmometer [3]. The paradox becomes particularly acute in the light of the chemiosmotic hypothesis of Mitchell [4]. Since proton translocations are conceived to be mediated by specific pumps embedded in a membrane with otherwise low proton conductance, presence of even a single gramicidin channel (pore radius ≈ 0.4 nm [5]) would discharge the postulated proton gradient [6]! Since the external osmolyte in mitochondrial studies is usually sucrose (0.53 nm radius [7]), the phenomenon of swelling can occur only if pores of larger radius are induced in normal mitochondria, leading to colloidal swelling (cf. Ref. 8). Can proton motive force exist across a membrane with large porosity?

This paradox closely parallels and is a historical consequence of yet another riddle which resulted in sucrose space hypothesis [9,10]. The paradoxical coexistence of rapid entry kinetics of sucrose during centrifugation into subcellular organelles [11], notably the mitochondria, which otherwise behave as perfect osmometers [3] led to the postulation of a two-compartment model, in which the intermembranous space was designated as the sucrose-permeable space and the matrix space as the sucrose-

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impermeable space [9]. Sucrose space measurements, based on the unverified dogma of sucrose space hypothesis, became central to current mitochondiral methodology, particularly with regard to the assessment of $\Delta\psi$ and ΔpH across the inner membrane [12,13]. With the advent of the chemiosmotic hypothesis that relies on sucrose space measurements to obtain the predicted values of $\Delta\bar{\mu}_{H^+}$ to quantitatively account for ATP synthesis, the original problem of mitochondrial swelling became secondary.

We initiated studies on a systematic reevaluation of the osmotic behaviour of mitochondria vis-à-vis swelling phenomena, since (i) swelling is primarily an osmotic phenomenon, (ii) the sucrose space hypothesis is now obsolete [14,15], and (iii) $\Delta\psi$ and ΔpH measurements based on sucrose space measurements with contingent centrifugal methodology are, therefore, suspect [16,17]. The experimental approach we adopted was based on the novel technique of enzyme osmometry [14–16], in which first-order kinetics of occluded/membrane-bound enzymes were linked to the robust physical theory of osmosis. In this paper, we demonstrate that the porosity of the mitochondiral inner membrane to polyols undergoes significant changes during oxidative phosphorylation as well as during high amplitude swelling, as evidenced by detailed osmometric studies. We characterized further the phenomenon of high amplitude swelling to account for the irreversible damage to mitochondria in terms of sequential and dynamic instability (and permeability) changes of respiring mitochondrial membranes in nonelectrolyte media.

Materials and Methods

Materials. Sucrose, mannitol, Tris, L-glutamic acid, L-malic acid, succinic acid, ADP, ATP, NADH, rotenone and hexokinase were obtained from Sigma, U.S.A. All other reagents were of analytical grade. Millipore filters were obtained from Millipore Corporation, U.S.A. [^{32}P]Orthophosphate (carrier-free) was obtained from Bhabha Atomic Research Centre, Bombay, India.

Methods. Isolation of rat liver mitochondria in 0.25 M sucrose, polarigraphic measurements of respiratory states, respiratory control ratio, ADP/O ratio and the osmometric analyses for the indices of oxidative phosphorylation, fumarase (L-malate hydro-lyase, EC 4.2.1.2) and high-amplitude swelling and protein estimations were carried out as described earlier [16].

Inorganic [^{32}P]phosphate incorporation into glucose 6-phosphate due to oxidative phosphorylation in mitochondria. The assay system comprised of 2 mM ADP/15 mM succinate (neutralized with Tris base directly)/10 mM potassium phosphate buffer (pH 7.4)/15 mM glucose/2 mM EDTA/5 mM $MgCl_2$/1.6 mg hexokinase/1.3 μCi carrier-free [^{32}P]phosphate per assay. The tonicity of medium was varied with sucrose. After equilibration of the reaction mixture with air for 10 min by gentle shaking, reaction was started by the addition of mitochondria (1.5 mg per assay volume of 0.5 ml). Reaction was stopped with 1.0 ml of an ice-cold (1 : 1) mixture of 4 M perchloric acid and 8% (w/v) ammonium molybdate. Radiolabelled glucose 6-phosphate, free of inorganic phosphate, was isolated into the aqueous phase, as described by Kagawa et al. [18] and the Cerenkov radiation was measured at 35% efficiency in a Packard Tri-carb liquid scintillation spectrophotometer (model 3255).

Enzyme osmometry. Activities of occluded enzymes, such as fumarase and membrane-bound enzyme systems such as the oxidoreductases, rates of respiration in various respiratory states, etc., were monitored under initial velocity conditions, as a function of varied external polyol concentration (i.e., osmolarity). It can be shown that the discontinuities in such osmotic profiles arise either due to lysis and release of the occluded enzyme, or due to hypotonic activation of membrane-bound enzyme systems/carrier proteins (cf. Refs. 14–16 for a detailed theoretical formalism, relevant definitions and methodological precautions). The break-point (i.e., discontinuity), obtained as the point of intersection of individual regression lines, is a measure of (i) the internal osmolarity, (ii) permeability to the external osmolyte and (iii) the elastic limits of the membrane.

Monitoring of high-amplitude swelling. Mitochondria were diluted into 3.0 ml 0.25 M sucrose with 10 mM Tris-HCl buffer (pH 7.4) at time, $t = 0$, and the changes in turbidity, defined as high amplitude swelling [1,2,19] were monitored at 520

nm; specific activity was defined as $\Delta A/\text{min}$ per mg protein. The maximal rate of swelling, $(dA/dt)\text{max}$, (obtained from digital readings at 20-s intervals) was shown to reflect volume changes in mitochondria vis-à-vis the external osmolarity. The osmometric profiles of swelling yield break-points of osmotic pressure-volume relationship directly, as described earlier [16].

Millipore technique to determine the dynamics of membrane instability during swelling. High-amplitude swelling was monitored spectrophotometrically as described above. Independent assay mixtures were filtered at 1-min intervals through 2.5 cm (diameter) Millipore filters of 0.45 μm pore size. The filtrates were collected into separate test-tubes, chilled rapidly to 0°C, and were assayed for marker enzymes subsequently. Protein concentration per assay was adjusted such that the filtration time did not exceed 10 s at each time-point. Total activity of relevant mitochondrial enzymes was assayed by prior solubilization of mitochondria in Triton X-100 such that the assay concentration of Triton X-100 did not exceed 0.15% (v/v). Specific activities of marker enzymes were defined as in legends to figures and tables.

Results and Discussion

Evaluation of the changes in the porosity of mitochondrial membranes in terms of permeability to polyols

The specific advantage in the measurement of reflection coefficients to polyols is in that the flux of a polyol (a hydrophilic nonelectrolyte) across the membrane depends solely on the concentration and mobility of the solute. In the case of ion fluxes, coexistence of electrical force complicates any direct interpretation of changes in the mobility of the ionic solute in the membrane [20]. The choice of polyols in our experiments was primarily restricted to sucrose and mannitol, since these are foreign, inert species for the mitochondrial membrane and specific proteinaceous channels are unlikely to exist [14,15]. An added advantage is the difference in the molecular mass, which would yield valuable information on the sieving capability of the membrane. The method of detection of variable mobility of these polyols in dynamic respiratory states depended on the inverse relationship between mobility of a solute across a membrane and the osmotic pressure exerted by that solute, i.e., the technique of osmometry. A rapid change of significant magnitude in the mobility of these rather large polyols, if detected, would be inconsistent with diffusion of the dehydrated solute molecules across the lipid bilayer, for thermodynamic reasons. Indeed, such changes would directly reflect changes in the fine structure of the inner mitochondrial membrane of a specific kind (cf., Ref. 15).

Osmolysis of mitochondria in sucrose and mannitol media

Mitochondria isolated in 0.25 M sucrose begin to lyse at approx. 0.1 M external sucrose [16]. The critical (i.e., break-point) external concentration of a specific osmolyte corresponding to the onset of lysis depends on its permeability across the membrane, under constant conditions of incubation. Since mitochondria possess two compartments, the release of corresponding marker enzymes, sulphite : cytochrome *c* oxidoreductase (intermembranous space marker) and fumarase (matrix space marker) were monitored in both sucrose and mannitol media of varying tonicity, at 15 min of incubation at 0°C. Data in Fig. 1 indicate the initial disruption of the outer membrane, followed by that of the inner membrane in mannitol media, as judged by break-point analysis of osmometric curves. Osmolysis in sucrose media, carried out simultaneously, yielded comparable break-points, as reported earlier [16]. These data suggest that the mitochondrial inner membrane is equally impermeable to sucrose and mannitol at 0°C.

Osmometry of high-amplitude swelling (Fig. 2)

Since the rate of change of turbidity yields a measure of the volume of the particle, we have earlier successfully utilized the phenomenon of high amplitude swelling to obtain the critical external osmolarities of sucrose media (i.e., 0.1–0.35 M), within which alone, mitochondria behave as perfect osmometers. An osmometric evaluation of rates of turbidity change at varying tonicity of the three polyols tested, yielded the rank order of break-points: sucrose < mannitol < glucose. As expected of the volume profile, the osmometric pro-

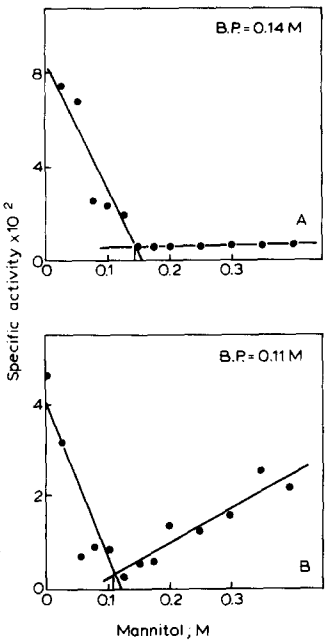

Fig. 1. Osmolytic behaviour of mitochondria isolated in 0.25 M sucrose. Release of marker enzymes into the medium is plotted against ambient mannitol concentration in the medium. (A) Sulphite: cytochrome *c* oxidoreductase. (B) Fumarase. Specific activities (μmol fumarate formed/min per mg protein (fumarase), μmol cytochrome *c* reduced/min per mg protein (sulphite: cytochrome *c* oxidoreductase) were expressed per mg protein in the incubation medium. Osmolytic behaviour in sucrose media was also assessed simultaneously and the corresponding break-points for lysis were (A) 0.15 M and (B) 0.15 M of external sucrose (figures omitted; cf., Ref. 16). Break-point (B.P.) analysis was carried out as described earlier [16].

files of high amplitude swelling in various media exhibited two break-points, one corresponding to the range of lysis (smaller break-point) and the other corresponding to the linear limit of osmotic contraction of the bilayer (larger break-point). While both the break-points appeared to exhibit a parallel shift with the polyol tested, the larger break-points alone satisfied the statistical criterion of a significant regression line connecting the break-points (henceforth referred to as the determinant slope).

A clear contradiction has emerged that the permeability of inner membrane to sucrose and mannitol was similar in osmolysis experiments, but different in turbidimetric studies. The difference could arise either due to the effect of temperature and/or due to the presence of respiration at 37°C as opposed to at 0°C.

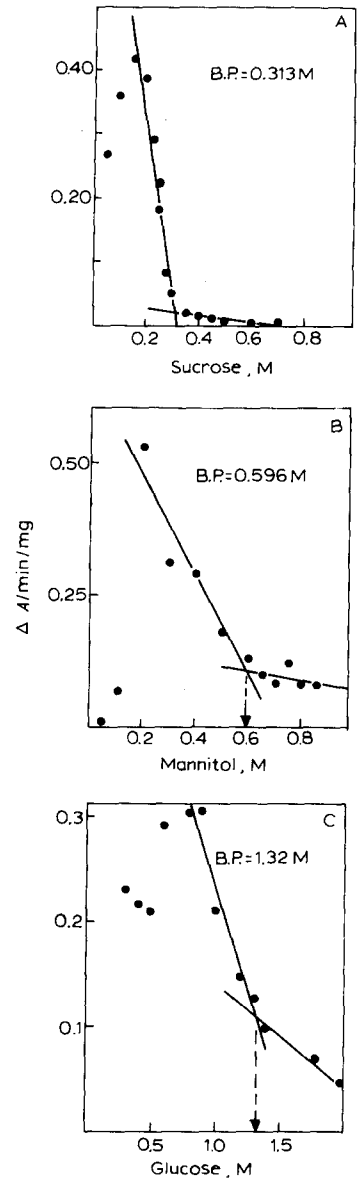

Fig. 2. Osmometric analysis of high amplitude swelling in mitochondria isolated in 0.25 M sucrose. Maximal rate of change in absorbance due to spontaneous swelling of mitochondria at 520 nm (ΔA/min per mg protein) is plotted as a function of ambient concentration of sucrose (A), mannitol (B) and glucose (C) in the assay medium. Break-point (B.P.) expressed as M solute concentration in the assay medium is indicated by a dashed arrow. Note: The break-point indicated in the text is the larger break-point ($B.P._1$) corresponding to deviation from the Boyle Van't Hoff osmotic pressure-volume relationship. The lower break-points ($B.P._2$) in the hypotonic range were not statistically evaluated for the reasons mentioned in the text ($B.P._2$ of 0.14 M for sucrose, 0.2 M for mannitol and 0.8 M for glucose, approximately).

We have compared the break-points of osmolysis of rat erythrocytes (since the erythrocyte membrane lacks the ability to respire) in sucrose, glucose and mannitol media, at varying times of incubation. The break-point of lysis was at 0.14 M external polyol (regardless of the polyol) at 15 min of incubation, which increased up to 0.16 M by 4 h for glucose only (data not given). Of these three polyols, an uptake mechanism is known to exist in eyrthrocytes for glucose only [21]. The erythrocyte experiments supported the possibility that respiration could be the determinant factor for the observed differences in reflection coefficients to these polyols.

Osmometry of fumarase

Osmotic titration of occluded fumarase activity was shown to exhibit a marked osmometric profile. Osmotic activation of the dicarboxylate transporter was shown to be responsible for the observed break-point of fumarase osmometry and not the release of the occluded enzyme on lysis. In a coupled assay system of an occluded enzyme and a transporter for the substrate, a break-point of enzyme osmometric profile larger than that for the osmolytic profile of the same occluded enzyme is diagnostic of hyposmotic activation of the transporter activity (cf., Ref. 16). Fumarase osmometry in sucrose and mannitol media (Fig. 3) clearly demonstrated that mannitol is more permeable (break-point > 0.8 M) than sucrose (break-point = 0.37 M) consistent with the turbidimetric data. An important aspect of fumarase osmometry was that it could be carried out at 10–15 min incubation time by a kinetic assay using a double-beam spectrophotometer with recorder or by prolonged (0.5 h or longer) end-point assays in individual test-tubes. The break-point concentration remained unchanged in sucrose media, tested by either of these methods, indicating that the osmometric methodology is sensitive to bulk changes in net osmolyte content only. In mannitol media, on the other hand, it was not possible to demonstrate any break-point by an end-point assay, since the activity levels were as high as those in the hypotonic range, even upto 0.75 M external mannitol (data not given). The instantaneous osmotic pressure, Π, required for constant tonicity by a permeable external solute, S, at time, $t = 1$ is:

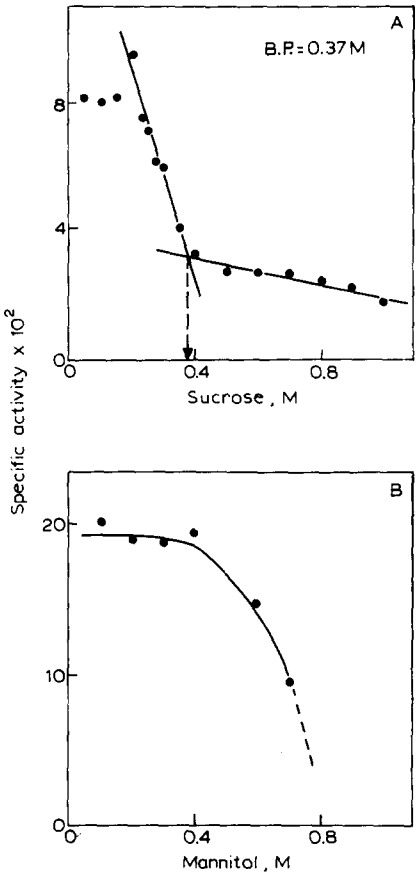

Fig. 3. Enzyme osmometry of the matrix enzyme, fumarase. Enzyme osmometry of fumarase was carried out as described earlier [16], in mitochondria isolated in 0.25 M sucrose. Specific activities were plotted as a function of sucrose (A) and mannitol (B) concentrations in the assay medium. Break-point (B.P.) analysis was carried out as described earlier [16]. $B.P._1$ in sucrose media is indicated while that in mannitol media is not discernible, for reasons described in the text.

$$\Pi^S(t=1) = \Pi^S(t=0) + RT\,\mathrm{d}s \int_{t=0}^{t=1} \mathrm{d}t \qquad (1)$$

where R is the gas constant and T the temperature. It necessarily follows that a time invariance of the break-point in sucrose media indicates its relative impermeability, compared to that of mannitol with demonstrable time variance.

Osmometric evaluation of oxidative phosphorylation

We reported earlier that state-3 respiration, respiratory control ratio, ADP/O ratio, fold stimula-

tion of latent ATPase activity by 2,4-dinitrophenol exhibit progressive inhibition with increasing tonicity of the external sucrose medium, using glutamate/malate as substrates [16]. This was the first experimental evidence that the stoichiometry of oxidative phosphorylation is not invariant, but depends on the physical state of the inner membrane. We reinvestigated the osmometric profiles of states of respiration with glutamate/malate as substrates in sucrose media using 17 concentrations of sucrose in the assays (Fig. 4) to confirm our earlier results. Essentially similar profiles were obtained with succinate as substrate as well (vide infra). The osmometric profiles deteriorated both in the hypotonic domain (corresponding to range of lysis) and in the range of hypertonic disruption (more than 0.4 M), with a volume dependence of indices of oxidative phosphorylation including stoichiometry (ADP/O ratio). Since both respiration (state 3) and ADP/O ratio were inhibited with progressive increase in the tonicity of the medium, phosphorylation should also be inhibited (as judged by esterification of inorganic phosphate). Indeed, $^{32}P_i$ esterification in a hexokinase-glucose coupled assay, using succinate as substrate, revealed a marked osmometric profile as expected (Fig. 5). Besides a high signal-to-noise ratio in these radioactive experiments, two break-points, one corresponding to the range of lysis and the other corresponding to the hypertonic domain, could be clearly seen. This was not possible in polarigraphic experiments for technical reasons. For statistical reasons of adequacy of number of data points, only the break-points corresponding to the range of lysis were evaluated.

The noise levels, besides the method itself, render measurements beyond a respiratory control ratio of 1.0 meaningless in polarigraphic measurements of oxidative phosphorylation. The break-points, corresponding to lysis and the linear limit of contraction, represent material properties of the system. Therefore, it follows that the critical external solute concentration corresponding to any defined and measurable physical state of the membrane (e.g., respiratory control ratio = 1.0) can be used in lieu of the break-points when necessary.

Indeed, this generality is of great importance since we can now assess variations in the reflection coefficients to polyols by osmometric methodol-

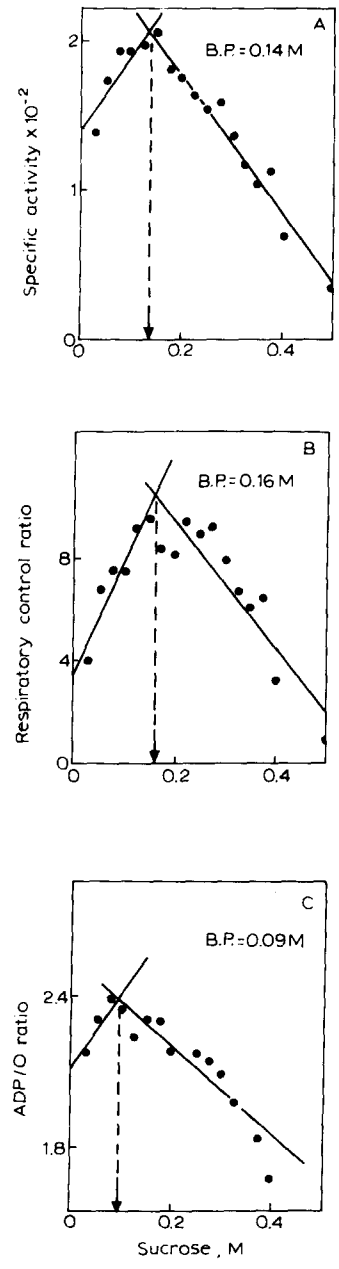

Fig. 4. Osmometric analysis of mitochondrial respiratory states. Rate of oxygen consumption (ng atoms oxygen consumed/min per mg mitochondrial protein) was determined polarigraphically in various respiratory states in mitochondria isolated in 0.25 M sucrose using glutamate+malate as substrate and plotted as a function of ambient sucrose in the assay medium. (A) State 3, (B) respiratory control ratio (rate of state 3/rate of state 4) and (C) ADP/O ratio (nmol ADP/ng atom oxygen consumed). Break-point (B.P.) expressed as M sucrose in the assay medium is indicated by the dashed arrow.

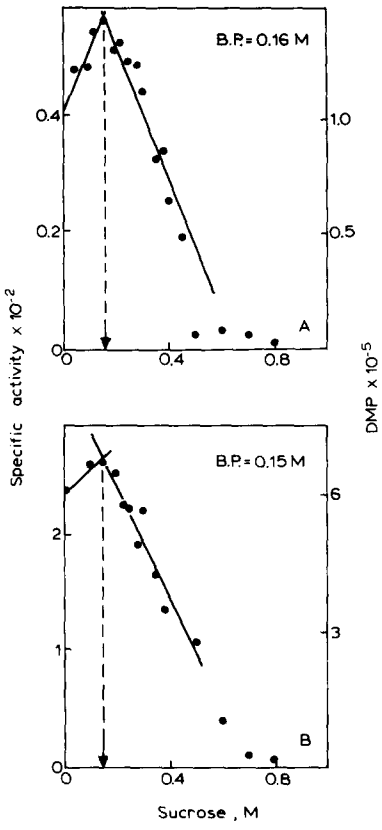

Fig. 5. Esterification of [^{32}P]orthophosphate to glucose 6-phosphate, in the absence of external substrate (A), in the presence of external substrate (succinate) (B), plotted as a function of ambient sucrose concentration. Break-point (B.P.) expressed as M sucrose in the assay medium indicated by the dashed arrow. Specific activity was expressed as nmol P_i esterified/min per mg protein. Total DPM accumulated for the time of incubation (10 min) is represented. The counts obtained at the break-point concentration of sucrose was 583-times the blank.

ogy, even in dynamic functional states. The classical methodology of assessment of reflection coefficients to solutes relies on methodology that require extrapolation to zero time [8], which is impossible in dynamic functional states.

Osmometric evaluation of oxidative phosphorylation in mannitol media (succinate as substrate) revealed an osmometric profile with a break-point in the range of lysis, comparable to that in sucrose media. However, respiratory control ratio remained above 1.0 even at the practicable limits of mannitol concentrations due to limited solubility (more than 0.8 M). It was puzzling to observe that the concentration of mannitol required to inhibit state 3 respiration, respiratory control ratio and ADP/O ratio were much larger than that of sucrose, though the range of lysis was the same for both sucrose and mannitol media (Fig. 6).

Since these detailed osmometric studies were the first of their kind and, since the shifts in break-points of oxidative phosphorylation were anomalous in hypertonic and hypotonic domains, a systematic comparison was made of the break-

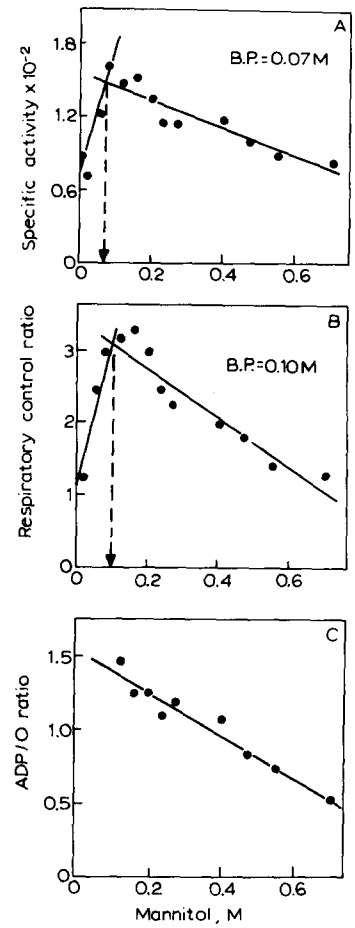

Fig. 6. Osmometric analysis of mitochondrial respiratory states. Rate of oxygen consumption (ng atoms oxygen consumed/min per mg) of mitochondrial protein was determined polarigraphically in various respiratory states using succinate as substrate in mitochondria isolated in 0.25 M sucrose (mitochondria were pretreated with rotenone, 1.0 μg/mg protein) and plotted as a function of ambient mannitol concentration. (A) State 3, (B) respiratory control ratio (rate of state 3/rate of state 4) and (C) ADP/O ratio (nmol ADP/ng atom oxygen consumed). B.P., break-point expressed as M mannitol in the assay medium.

points of the indices of oxidative phosphorylation as well as respiratory states in sucrose and mannitol media, using glutamate/malate and succinate as substrates (Table I). It may be recalled that state-2 and state-4 respiration exhibit a single break-point in the lysis range due to respiratory control [16]. The data conclusively showed that the break-points corresponding to the range of lysis in mannitol media were comparable to those in sucrose media. On the other hand, in turbidimetric data, the larger and the smaller break-points uniformly increased with increasing permeability of the external solute, as would be expected from the physical theory of osmosis.

The anomalous nature of osmometric profiles in oxidative phosphorylation

As the permeability to external solute increases, the two break-points should be displaced on the osmolarity scale in the same direction proportionately. This can be seen in the osmolytic profiles of rat erythrocytes, using NaCl, LiCl and NH_4Cl media of varying tonicity (Fig. 7A). It should be noted that the two break-points in erythrocyte osmolysis curves pertain to the hypotonic domain only, due to intrinsic osmotic heterogeneity, and should not be confused with the two break-points in volume-related profiles corresponding to both hypo- and hypertonic domains. The direction of shift in break-points can be shown to be dependent also on whether the net internal solute is gained or lost. Addition of valinomycin leads to selective loss of internal K^+ [22], while gramicidin S enhances permeability to external Na^+ as well [23], conferring osmotic protection and lability of erythrocytes, respectively. Indeed, such was the case as shown in osmolytic profiles of ionophore-treated erythrocytes (Fig. 7B). The anomalous osmometric behaviour of oxidative phosphorylation is summarized in Fig. 7C, in that, a permeable solute resulted in a shift of the larger but not the smaller break-point (corresponding to curve C). A solution to this paradox lies in the recognition that the centrifugal isolation of mitochondria in 0.25 M sucrose would result in sucrose being the major osmolyte in the matrix space. The rate of respiration increases with decrease in tonicity, such that, at enhanced rates of respiration corresponding to state 3 (more than 100 ng atoms oxygen/min per mg protein), the induced porosity of the inner membrane could be large enough to lead to a loss of internal sucrose as well. This would result in a leftward shift of the break-point corresponding to lysis from that expected from the osmotic theory, as in the case of valinomycin-treated erythrocytes. We also assessed the range of rates of respiration associated with high amplitude swelling (4–9 ng atoms oxygen/min per mg protein) and fumarase osmometry (6–10 ng atoms oxygen/min per mg protein). In

TABLE I

OSMOMETRIC ANALYSIS OF MITOCHONDRIAL RESPIRATORY STATES AND INDICES OF OXIDATIVE PHOSPHORYLATION

Osmometric analysis of mitochondrial respiration was carried out as described in Materials and Methods. Plots of the specific activity of respiratory states against external solute concentration yield osmometric profiles (cf., Fig. 4 and Ref. 16). Only the smaller break-point corresponding to the hypotonic region of lysis is given in the table. Respiratory control ratio (RCR) obtained in the hypertonic region, larger break-point, is 0.4 M in sucrose media and approx. 0.7 M in mannitol media, with either glutamate + malate or succinate as substrates. Succinate oxidation were carried out with mitochondria pretreated with rotenone (1.0 μg/mg mitochondrial protein).

Medium	Substrate	Break-point concentrations of external polyol (M)				
		Respiratory states			RCR	ADP/O
		2	3	4		
Sucrose	glutamate + malate	0.06	0.14	0.075	0.16	0.09
Sucrose	succinate	0.07	0.21	0.102	0.155	0.09
Mannitol	glutamate + malate	0.09	0.13	0.12	0.13	0.12
Mannitol	succinate	0.1	0.07	0.12	0.1	< 0.1

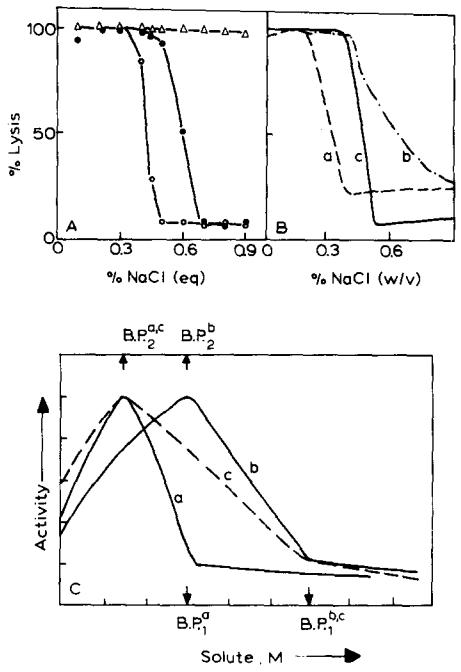

Fig. 7. Osmolysis of rat erythrocytes (A and B). Osmolytic profiles of rat erythrocytes were obtained as described earlier [16]. Percentage lysis was calculated taking the absorbance at 540 nm of the supernatants of cells suspended in water. (A) Osmolysis of rat erythrocytes in various electrolyte media. The abscissa is represented as % (w/v) NaCl equivalents for the equimolar media of NaCl (○), LiCl (●) and NH_4Cl (△). (B) Osmolysis of rat erythrocytes in varying concentrations of NaCl media. Erythrocytes were pretreated with $1 \cdot 10^{-6}$ M valinomycin (a); $1 \cdot 10^{-5}$ M gramicidin S (b) and control (c). Note the presence of two discontinuities (break-points) in osmolysis profiles of rat erythrocytes, one corresponding to the onset and the other corresponding to completion of lysis. 19 data points for each curve, omitted for clarity. (C) Anomalous shifts in break-points of osmometric profiles. Given two solutes with osmometric profiles a and b (such that the latter solute is twice as permeable as the former), the osmometric profiles of activity would be as indicated by the solid lines. Note that both the break-points $B.P._1$ and $B.P._2$ shift proportionately. An anomalous situation could arise, as in the case of profile c. Of the two break-points of profile c, $B.P._1$ is larger than that of curve a but $B.P._2$ is same as that of a. Since a shift to right indicates increased permeability to external solute, the anomalous behaviour may be interpreted as a shift to left (compared to profile b) due to increased permeability to internal solute, as a function of the activity (e.g., respiration).

both instances, the shifts in break-points of the osmometric profiles were as expected from theoretical considerations, indicative of enhanced permeability, predominantly to mannitol only. Thus, with increasing tonicity of the medium, both respiration and respiration-induced porosity of the inner membrane would be progressively inhibited such that, the magnitude of inhibition (and therefore the large break-point) would exclusively depend on the permeability to external solute. The 'beneficial' effect of mannitol in studies on oxidative phosphorylation [24] clearly emerges from its greater permeability, since a permeable solute would necessarily be hypotonic.

A critical comparison of the above five classes of experiments are consistent with (1) the relative impermeability of the mitochondrial membrane to polyols in the nonrespiring state, (2) enhanced porosity of the mitochondrial inner membrane to mannitol at low levels of respiration and (3) larger changes in the porosity of the inner membrane with increasing rate of respiration incidental to oxidative phosphorylation, sufficient to lead to exchange of bulk sucrose across the inner membrane.

Since high amplitude swelling represents dramatic changes in the volume of mitochondria resulting in irreversible changes in mitochondrial structure and function, any osmotic explanation for mitochondrial swelling would be incomplete unless the dynamics and mechanism of high amplitude swelling are understood. On a methodological plane, such studies would be critical to validate the popular use of turbidimetric studies on volume regulation in mitochondria.

Studies on high-amplitude swelling

Extensive, though unresolved, documentation exists in literature that dilution of mitochondrial protein to a concentration below 1 mg/ml in isotonic sucrose media result in large decrease in turbidity reflecting parallel increase in volume [1,2,19,25,26]. The properties of the phenomenon are: (1) rotenone sensitivity; (2) initial time delay followed by nearly exponential decay of turbidity; (3) insensitivity to rotenone after the onset of swelling; (4) marked sensitivity to external solutes, particularly electrolytes; (5) uncoupled nature of the swollen mitochondria, which do not contract on addition of ATP. Since respiration of endogenous substrates is necessary to induce high-am-

plitude swelling, we initially compared the time-course of respiration with that of turbidity changes.

Interrelationship bteween the time delay of high-amplitude swelling and respiration

Mitochondrial protein was adjusted to yield a relatively smooth profile of turbidity change, such that the time delay of high-amplitude swelling could be critically measured by plotting dA/dt as a function of time (Fig. 8A). Similarly, oxygen consumption was monitored polarigraphically under identical conditions of assay simultaneously. At several protein concentrations tested, respiration invariably exhibited an abrupt decrease at defined time intervals (time delay of respiration) (Fig. 8B). Regardless of the protein concentrations employed (which enhanced both the time delay of high-amplitude swelling and of respiration), these events occurred nearly simultaneously. Fig. 8C illustrates the linear correlation between the time delay of high-amplitude swelling and of respiration at various protein concentrations. Depletion of endogenous substrates would not account for the abrupt decrease observed in respiration, since endogenous substrates cannot be diluted, while the time delay of respiration decreased with dilution of protein. Addition of exogenous malate only enhanced the time delay but did not abolish the phenomenon of high amplitude swelling (Fig. 8D). The mitochondria were insensitive to rotenone or external malate beyond the time delay of high-amplitude swelling/respiration, suggesting a specific membrane event at this time-point.

The reported loss of matrix constituents such as NAD^+, Mg^{2+} [2,26] would account for the insensitivity of mitochondrial respiration to malate beyond the time delay of high-amplitude swelling/respiration. We therefore systematically investigated the dynamics and magnitude of the instability of the outer and inner mitochondrial membranes, by monitoring the leak of specific marker enzymes, as a function of incubation time under conditions of high amplitude swelling (also monitored spectrophotometrically).

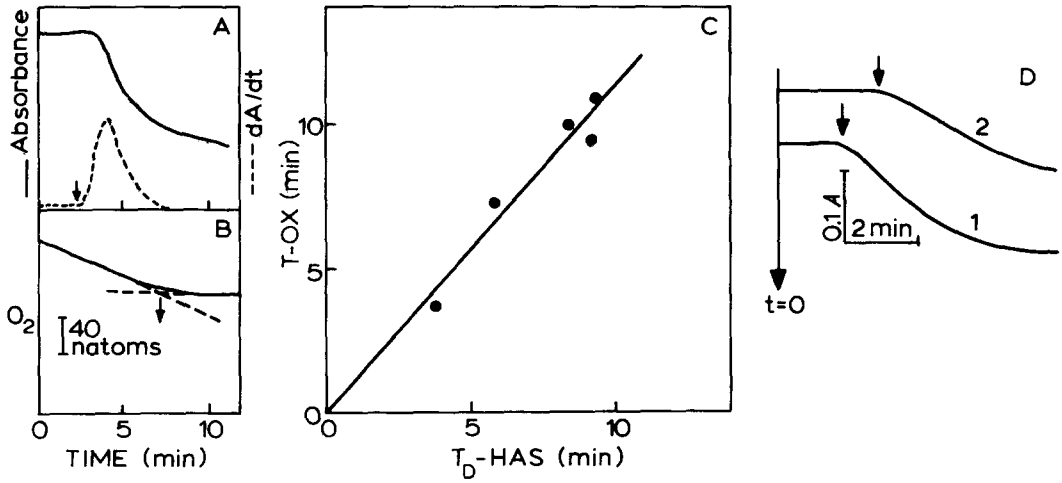

Fig. 8. Interrelationship between the time delay of high-amplitude swelling (T_D-HAS) and the time delay of respiration (T-OX) (A) A schematic diagram of the time-course of swelling and dA/dt plotted as a function of time. Note that the time delay of high-amplitude swelling is clearly identified (by an arrow) using the derivative. The peak, (dA/dt)max, is used to evaluate specific activity. (B) Time-course of oxidation of endogenous substrates under conditions of high amplitude swelling by polarigraphy (cf. Fig. 2). The polarigraphic tracing represented here was obtained at 1.3 mg mitochondrial protein/ml assay medium, resolved into two domains of linear rates of oxidation to obtain the time delay of respiration, the time at which a discontinuity in the rate of oxidation was observed. (C) Correlation between the time delay of high-amplitude swelling and of respiration at various protein concentrations. The time delay of high-amplitude swelling and of respiration were measured at various protein concentrations as in A and B. Coefficient of correlation of the regression line was 0.97 ($n = 5$; $P < 0.05$); (D) Effect of addition of exogenous substrate, (Tris) malate, 6.7 mM (pH 7.4) on the time-course of swelling. (1) Control; (2)+malate. Arrows indicate the time delay of high-amplitude swelling (122 μg mitochondrial protein/ml assay medium).

Dynamics of instability of mitochondrial membranes during high-amplitude swelling

Release of the intermembranous marker enzyme, sulphite : cytochrome *c* oxidoreductase coincided with the time delay of high-amplitude swelling/respiration as shown in the composite profile in Fig. 9. The onset of release of the matrix enzyme, fumarase, could be critically distinguished to occur after the release of the intermembranous enzyme. The maximal rate of swelling occurred at a time-point intermediate to the onset of instability in the outer and inner membranes. Release of protein exhibited a composite profile.

The observed decrease in the release of marker enzymes at longer intervals could be attributed only to variable susceptibility of mitochondrial membranes to Millipore filtration during swelling. A parallel experiment using centrifugal methodology for phase separation also yielded a profile of release of marker enzymes as in the case of Millipore filtration (data not given). The Millipore data alone was adequate to critically distinguish the onset of various events. Thus, the data clearly indicated that the outer membrane disrupted first, leading to cristal unfolding, which in turn contributed to maximal turbidity change. Then the inner membrane also disrupted. Since respiration abruptly ceased at a time-point corresponding to the time delay of high-amplitude swelling, respiration per se could not account for the lysis of the inner membrane. We could observe linear negative correlation between the rate of turbidity change $((dA/dt)max)$ and log buffer (or log protein) concentrations, indicating that ionic strength of the external medium could affect swelling (data not given). Since high amplitude swelling was primarily demonstrated in sucrose media (i.e., nonelectrolyte media), presence of unscreened fixed charges on the mitochondrial membranes could account for an instability of the membranes by the field created by surface charge density. Since surface charge could be selectively modified by specific ionic surfactants, we investigated the release of marker enzymes in variously treated mitochondria.

Data in Table II shows that rotenone, antimycin A and sodium dodecyl sulphate (SDS), an anionic detergent, did not prevent, while cetyltrimethylammonium bromide (CTAB), a cationic detergent, inhibited the release of the intermembranous marker enzyme. The release of fumarase was inhibited by both the detergents of opposing

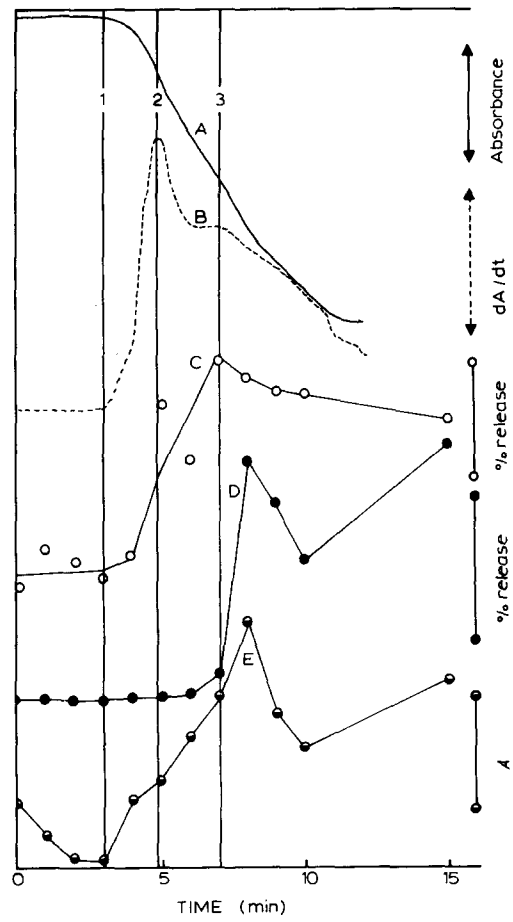

Fig. 9. Time-course of high-amplitude swelling vis-à-vis the release of marker enzymes from the intermembraneous and matrix spaces of mitochondria. The time-course (A) was recorded on addition of 560 μg mitochondrial protein/ml assay medium (0.25 M sucrose with 10 mM Tris-HCl (pH 7.4)) at zero time. The derivative dA/dt was computed (cf. Fig. 8A) and plotted as a function of time (B). Percentage release of the marker enzymes and protein was assayed as a function of time, as described in Materials and Methods. Percentage release of sulfite: cytochrome *c* oxidoreductase (C), of fumarase (D), and of protein (E). The vertical bars with arrows on the right margin depict units: (A) 0.1 *A*, (B) 0.053 ΔA/min per mg protein, (C) 20% of total mitochondrial sulphite: cytochrome *c* oxidoreductase, (D) 10% of total mitochondrial fumarase, (E) 0.1 *A* at 280 nm (protein). Vertical lines represent critical time-periods, to facilitate comparison in the composite graph: (1) the time delay of high-amplitude swelling, (2) $(dA/dt)_{max}$ and (3) onset of fumarase release. The data points in C, D and E were means of duplicate assays.

TABLE II

RELEASE OF SULPHITE:CYTOCHROME *c* OXIDOREDUCTASE AND FUMARASE FROM MITOCHONDRIA AT THE COMPLETION OF SWELLING

Swelling was monitored spectrophotometrically and release of enzymes was measured at 95% of total turbidity change, i.e. at 15 min in Expt. 1 and at 25 min in Expt. 2. Therefore, comparisons should be made only within each experiment. 10 mM Tris-HCl (pH 7.4) was present in all assays. The released activity was expressed as % of total activity in mitochondria per assay, obtained on treatment with 0.15% (v/v) Triton X-100. All measurements were in duplicate; the data reported were the mean values. Mitochondria were preincubated with rotenone (0.7 μg/mg protein) and antimycin A (1.18 μg/mg protein) for 10 min at 0°C, before addition. Mitochondria were directly suspended in media containing $1.04 \cdot 10^{-5}$ M SDS or $2.7 \cdot 10^{-5}$ M CTAB. Figures in parentheses indicate corresponding enzyme release at zero time. n.d., not detected. In both experiments, the assay medium concentrations were: sucrose, 0.25 M; NaCl, 0.125 M; KCl, 0.125 M.

Assay medium	Treatment	Percentage release	
		Sulphite: Cyt *c* oxidoreductase	Fumarase
Experiment 1			
sucrose	–	38.2 (8.4)	18.8 (0.8)
sucrose	rotenone	31.3	1.25
sucrose	antimycin A	21.2	1.28
sucrose	SDS	46.3	6.88
sucrose	CTAB	6.75	1.03
Experiment 2			
sucrose	–	33.4 (8.38)	42.81(3.86)
sucrose	rotenone	18.1	7.00
NaCl	–	6.34(4.98)	10.2 (2.23)
NaCl	rotenone	5.34	3.62
KCl	–	5.86(4.48)	19.2 (n.d.)
KCl	rotenone	5.79	3.39

charge, as would be expected of the stabilziing (protective) action of an intercalating agent [27]. Presence of isotonic electrolyte media (NaCl, KCl), which would screen the fixed surface charges, inhibited release of either enzyme. These results show that, lack of realization that mitochondria swollen by different agents under different incubation conditions would exhibit different dynamics of instability, prevented an understanding of the mechanism and the dynamics of high amplitude swelling thus far.

If the presence of unscreened fixed charges contributes to swelling, addition of intercalating agents at any time-point after the time delay of high-amplitude swelling should promote/reverse the turbidity changes in a predictable manner. Indeed, this was the case as shown in Fig. 10. CTAB reversed the turbidity decrease whereas SDS promoted turbidity decrease at all time-points. Addition of these ionic detergents at zero time also had comparable effects on the dynamics as well as the time delay (data not given). On the other hand, increase in external osmolarity to 0.5 M sucrose after the time delay of high-amplitude swelling, arrested but not reversed the changes in turbidity due to swelling (data not given), thus supporting the osmotic nature of the swelling of mitochondria in which surface charge density plays an important role. The charge-specific effects of ionic surfactants on turbidity further confirm the dominant contribution of membrane aggregation/

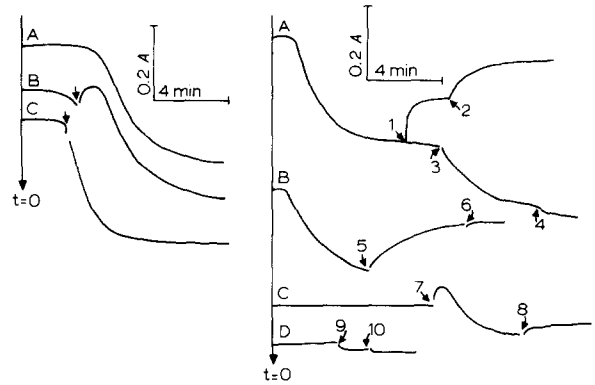

Fig. 10. Effect of addition of ionic detergents subsequent to the addition of mitochondrial protein (850 μg/ml assay medium added at time, $t = 0$). Left: detergents added at the time delay of high-amplitude swelling (indicated by arrows). (A) Control, (B) CTAB ($2.7 \cdot 10^{-5}$ M in the assay medium) and (C) SDS ($1 \cdot 10^{-5}$ M) in the medium. Right: effect of sequential addition of detergents and Mg^{2+} after near completion of swelling, at times indicated by arrows. (A) Addition of CTAB ($2.7 \cdot 10^{-5}$ M) sequentially (1 and 2) and SDS ($1 \cdot 10^{-5}$ M) sequentially (3 and 4). The time-course of swelling prior to addition of detergent was identical and therefore three independent curves (including control) were superimposed. (B) Effect of addition of Mg^{2+} (20 mM) sequentially (5 and 6). (C) Effect of sequential (7 and 8) addition of CTAB ($4 \cdot 10^{-5}$ M) to mitochondria pretreated with rotenone (1.0 μg/mg mitochondrial protein). (D) Effect of sequential addition (9 and 10) of SDS ($1 \cdot 10^{-4}$ M) to rotenone-treated mitochondria. Profiles similar to C and D were obtained when mitochondria were pretreated with antimycin A (1.2 μg/mg protein, data not given).

disaggregation phenomena (e.g., crystal unfolding) to changes in turbidity during high amplitude swelling.

These experiments were consistent with initial swelling of mitochondria due to enhanced porosity of the membrane caused by respiration as well as unscreened fixed charges on the surface of membranes suspended in nonelectrolyte media. The disruption of outer membrane appeared to be primarily mechanical. Removal of the mechanical constraint of the outer membrane led to crystal unfolding as well as sustained swelling, primarily driven by surface charge density. The disruption of the mitochondrial inner membrane appeared to be an extreme case of enhanced porosity in nonelectrolyte media.

Detailed studies on the characterization of the uncoupled swollen mitochondira would be published elsewhere. Briefly, evidence was obtained that the swollen mitochondria consist of a mixture of resealed, normally oriented mitochondria (osmotically active) and open fragments (osmotically inactive).

Concluding remarks

Instantaneous changes in the reflection coefficients to the relatively large polyols, sucrose and mannitol, during oxidative phosphorylation in normal functional mitochondria offers a unique and novel approach to probe the fine structural alterations in the inner membrane on energization. This approach is based on the irreversible thermodynamic description of a 'pore', with the significant difference that changes in reflection coefficients to polyols ($\Pi_{observed}/\Pi_{theoretical}$) were assessed relatively rather than by convential reference to water fluxes [8]. Since the flux of an inert polyol (and hence the reflection coefficient):

$$J_s = U C \frac{dC}{dx} \tag{2}$$

where U is the mobility, C the concentration and dC/dx the driving force of concentration gradient [20], rapid changes in the flux of these solutes would imply existence of pores of larger radius (more than 0.53 nm for sucrose).

Two observations of fundamental importance emerge from these osmometric studies on oxidative phosphorylation and high-amplitude swelling. The first pertains to the generality of hypoosmotic activation of respiration and other enzyme systems and indices, which may be exemplified as:

$$J_{ox} = J_{ox}(max) - \tilde{K}\Pi \tag{3}$$

where J_{ox} is the rate of oxidation, $\tilde{K}$ an empirical 'elastic' constant and Π the external osmotic pressure. While the physical basis of osmotic activation of membrane-bound enzyme systems is not yet clear, $\tilde{K}$ of a membrane-bound enzyme appears to emerge from the 'strain' developed within the membrane as a consequence of volume change (or osmotic stretch, based on a spherical approximation of the particle). $\tilde{K}_{respiration}$ appears to be a useful phenotypic marker for osmotolerance in microbes and plants (Rao, N.M., and Sitaramam, V., unpublished data). The second important observation pertains to the variation in the mobility (U) of the inert polyols with J_{ox} as well as with surface charge density, $\delta\sigma$, (which, in turn, is influenced by J_{ox} [28]), such that:

$$U = f(J_{ox}, \delta\sigma) \tag{4}$$

Together, these observations account not only for the observed osmometric profiles, but also for the observed shifts in break-points in terms of net influx or efflux of osmolytes in vitro, (cf., Fig. 7) as well as the observed volume fluctuations in vivo [29,30].

Experimental demonstration of variable porosity to polyols during oxidative phosphorylation is incompatible with the chemiosmotic hypothesis both conceptually and methodologically. At steady state:

$$J_{ox} n = L \Delta p \tag{5}$$

where $n = H/O$; L the inward proton leak and Δp the protonmotive force [31]. The chemiosmotic hypothesis assumes that n and the low phospholipid proton conductance component of L are invariant. Osmotic susceptibility of ADP/O ratio directly shows that n is not a constant. Rapid permeation of polyols during oxidative phosphorylation in normal mitochondira would be incompatible with an invariant proton conductance of the lipid bilayer and it would be absurd to invoke the

ATPase channel per se for polyol permeation. Existence of Δp or its variants of localized proton gradient hypotheses [6,32] would be incompatible with a membrane highly permeable to these relatively large polyols, regardless of whether water in such pores is structured or not [33].

On a methodological plane, variable porosity induced by respiration indicates that $\Delta\bar{\mu}_{H^+}$ measurements could be in serious error. In fact, equilibration of external sucrose with the matrix space during oxidative phosphorylation (as we have already demonstrated with gravity-mediated entry of sucrose) would necessarily mean that the true $\Delta\bar{\mu}_{H^+}$ could be too low to account for ATP synthesis. Additional evidence of direct measurements and localization of [^{14}C]sucrose in the matrix space during oxidative phosphorylation would be desirable. The uniqueness of osmometric methodology is not only in circumventing the specific methodological problems associated with localization of sucrose in the matrix space [14–17], but also in its noninvasive approach to assess the validity of chemiosmotic hypothesis in normal mitochondria, unlike the earlier attempts involving impaling by microelectrodes [34] or high pressure disruption to obtain open fragments [35].

Variable porosity to polyols induced by energization of membranes is by no means selective to oxidative phosphorylation or gravitational field (centrifugation), since comparable changes were also found during action potentials [36] and in erythrocytes on suspension in nonelectrolyte media, due to surface charge density emerging from unscreened surface charges (unpublished data). These observations suggest existence of density fluctuations in the lipid domain on energization of biological membranes by diverse means, of which oxidative phosphorylation could be a special case.

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